Neuron, Vol. 28, 1–9, October, 2000, Copyright ©2000 by Cell Press

The Last Shall Not Be First: The Ordered Generation of Progeny from Stem Cells

Discussions of CNS stem cell biology often leave the impression that neural stem cells persist throughout life and that stem cells from the adult cortex are the same as the stem cells that build the CNS during fetal development. In fact, the properties of CNS stem cells have not yet been carefully compared throughout ontogeny; however, by analogy to stem cells of the immune system, we would expect neural stem cells to change their properties over time. Fetal hematopoietic stem cells (HSC) have a broader developmental potential than adult HSCs in that they are able to make certain classes of T cells that are not made by adult HSCs, even when the adult HSCs are transplanted into the fetal environment (Ikuta et al., 1990). Apart from such overt changes in potential, HSCs also change their properties in more subtle ways with age. Adult HSCs are less able to produce certain types of B cells and erythrocytes than fetal HSCs (Wood et al., 1985; Kantor et al., 1992). Even the cell cycle status and proliferative potential of HSCs change throughout ontogeny. Therefore, in considering neural stem cell biology, we must allow for the possibility that CNS stem cells, like HSCs, can change their properties with time or changes in location and that these changes in properties may regulate their function in vivo. In this issue of *Neuron*, Qian, Shen, Temple, and colleagues (2000) have started to illuminate subtleties in neural stem cell biology that suggest that CNS stem cells are not all created equal and that their intrinsic properties change over time.

Neural Stem Cells Change Over Time

Neurogenesis consistently precedes gliogenesis in both central nervous system (CNS) and peripheral nervous system (PNS) development. Qian, Shen, and Temple now show that a program that brings about neuronal differentiation before glial differentiation is encoded intrinsically within CNS stem cells. They cultured individual cells from the E10 cortex (which corrresponds to the period of neurogenesis in the mouse) at clonal density and then followed the proliferation and differentiation of individual clones by time-lapse video microscopy. In doing so, they could retrospectively construct a "family tree" for each stem cell clone. Multipotent progenitors in culture always generated neuroblasts before glioblasts; furthermore, multipotent progenitors from the E10 cortex gave rise to many more neurons than did multipotent progenitors from the E16 cortex even though there was no obvious difference in their capacity to generate glia. Thus, in addition to encoding the order of neurogenesis/gliogenesis, stem cells also change over time in their propensity to generate neurons, despite remaining multipotent.

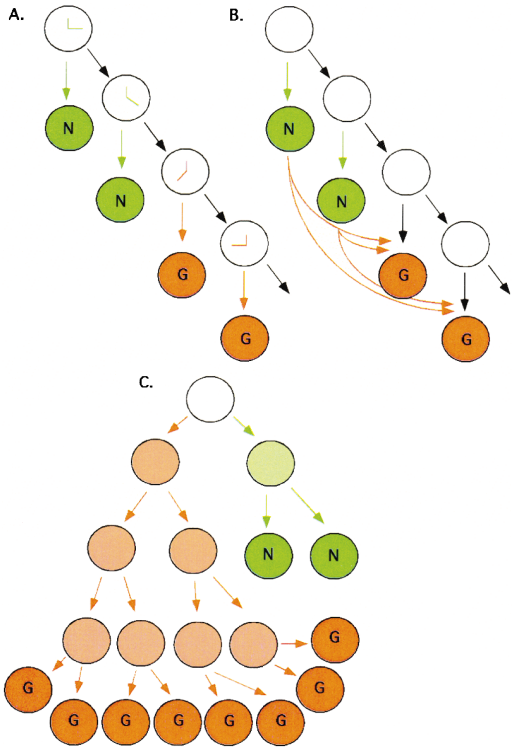
How Stem Cell–Intrinsic Is the Neuron-First Program?

The demonstration that information required for the generation of neurons before glia is intrinsic to individual stem cell–derived clones poses the question of mechanism. One possibility is that there is a clock intrinsic to stem cells that determines when daughter cells will be specified to adopt neuronal fates and when they will adopt glial fates (see figure, panel A). But as acknowledged by Temple and colleagues, there are a number of variations on this theme that would allow environmental signals or feedback mechanisms to also modulate the process. For example, it is possible that stem cells are programmed to generate neurons first but that a feedback signal from the neurons to the stem cells induces glial specification (see figure, panel B). In addition to the order of specification, differences in the proliferative behavior of restricted neuronal and glial progenitors may also contribute to the generation of neurons followed by glia. For instance, neuronal and glial specification could occur at the same time but neuroblast differentiation may occur much faster than glioblast differentiation (see figure, panel C). The important point is that the timing of differentiation may be regulated in part by controls acting on the proliferation of restricted progenitors as well as by controls on stem cell function. Thus, while a program that causes neurons to differentiate before glia may be intrinsic to individual stem cell clones, this does not necessarily mean the whole program acts intrinsically within stem cells: aspects of the mechanism may depend upon feedback regulation or effects on restricted progenitors.

Possible Stem Cell–Intrinsic Controls on Specification

What sorts of cell-intrinsic mechanisms could lead to the specification of neuroblasts first and glioblasts second? There may be internal mechanisms that measure time or the number of self-renewing divisions of cortical stem cells and that cause early asymmetric divisions to generate neuroblasts, leaving later divisions to generate glioblasts (see second figure). This would explain the generation of neurons first, the reduced number of neurons produced by E16 stem cells, and the presence of some multipotent progenitors among the glioblasts that continue dividing after the initial four rounds of division in certain clones. Such a mechanism would require a way to link specification decisions to time or to the mitotic history of a stem cell lineage. Candidates for stem cell–intrinsic changes that might be linked to cell-specification decisions include changes in telomere length (see figure, panel A), or the level of cyclin-dependent kinase inhibitor p27 (see figure, panel B), or the level of growth factor receptors (see figure, panel C).

In the case of the latter, prior studies have implicated several types of receptors whose expression levels change within lineages over time and that could be linked to cell-specification decisions by stem cells. Burrows, Lillien, and colleagues (Burrows et al., 1997) demonstrated that EGF receptor expression increased over time in cortical stem cells and that increasing EGF re-

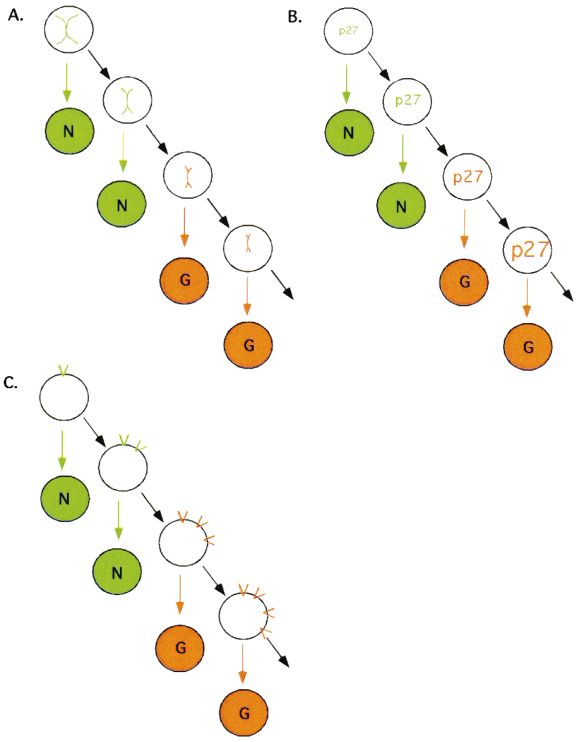


Models for How Stem Cells Generate Neurons before Glia

(A) Stem cells may contain a cell-intrinsic clock that counts time or cell divisions and that determines whether neuroblasts or glioblasts are produced as a result of each cell division. Early asymmetric divisions would give rise to neuroblasts and later divisions would give rise to glioblasts. In this way, neurons would be generated first, and more neurons would be generated by stem cells at earlier stages in development. This mechanism would be entirely stem cell intrinsic.

(B) Stem cells may be programmed to generate neurons first, but the generation of glia may depend upon a feedback signal from neurons. A series of recent studies has suggested that Notch activation in both CNS stem cells (Furukawa et al., 2000; Gaiano et al., 2000) and neural crest stem cells (Morrison et al., 2000) can promote glial specification. Thus, Notch ligand expression by neuroblasts might activate Notch on stem cells to promote glial differentiation.

(C) Some stem cell divisions might generate glioblasts and neuroblasts at the same time, but the neuroblasts may differentiate before glia because glioblasts proliferate for longer before differentiating. Multipotent progenitors are not colored, while neuroblasts are green and glioblasts are red. The model cannot fully explain the behavior of stem cells from the early cortex but may describe the behavior of multipotent progenitors from late fetal development or within clones that have already been through several rounds of division in vitro.



Possible Stem Cell–Intrinsic Clocks that Might Count Mitoses

If there is a stem cell–intrinsic clock as postulated in the first figure, panel A, what might it be?

(A) The division history of stem cells may be marked by changes in telomere length, and telomere length could be coupled to cell-specification decisions such that long telomeres cause neuroblast specification while shorter telomeres cause glioblast specification. But there is not yet any evidence that cells can sense relatively small changes in telomere length and couple such changes to cell fate decisions.

(B) Intracellular regulatory molecules may accumulate over time with each cell division, and the levels of such factors may be coupled to cell fate decisions. For example, part of the timer that determines when oligodendrocyte precursors stop proliferating and differentiate is the gradual accumulation of the cyclin-dependent kinase inhibitor p27 (Durand et al., 1997). Accumulation of p27 in stem cells could be associated with reduced self-renewal potential and increased propensity for glioblast specification.

(C) Cell surface expression of growth factor receptors by stem cells may gradually increase with each cell division they undergo. Increased expression of these receptors may be associated with increased sensitivity to environmental factors that specify gliogenesis rather than neurogenesis. Candidates for such cell surface receptors include the FGF, EGF, and thyroid hormone receptors (see text).

sponsiveness was associated with an increased propensity to generate glia. Qian, Shen, and Temple also documented an increased responsiveness of cortical progenitors to EGF after they had been cultured for 7 days. Similarly, this group had previously reported that FGF concentrations increased in vivo in late fetal development and that higher FGF concentrations in culture promoted glial differentiation (Qian et al., 1997). Finally, expression of thyroid hormone receptors has been shown to increase over time in oligodendrocyte precursors (Gao et al. 1998), and thyroid hormone has been shown to promote glial differentiation by CNS stem cells (Johe et al. 1996). In each of these cases, receptor expression levels could increase over time or with each stem cell mitosis, reducing the threshold for response to growth factors and increasing the probability of glial differentiation. However, in order to explain the switch observed in culture, the cells themselves would have to have produced EGF, FGF, or thyroid hormone.

Future Questions

We are accustomed to thinking in terms of stem cell potential: a cell either has the potential to make neurons or it does not. Qian, Shen, Temple, and colleagues illustrate the complexity of stem cell biology by showing that stem cells can gradually make quantitatively fewer

neurons over time without losing the qualitative ability to generate neurons. One of the next steps will be to determine if there are quantitative or qualitative changes in the *types* of neurons that a stem cell lineage can make over time. One suggestion that this might be the case comes from the work by McConnell and colleagues who showed that there are intrinsic restrictions in the ability of cortical progenitors to give rise to neurons in different levels of the cortex with increasing developmental age (Desai and McConnell, 2000).

In addition, while Qian, Temple, and colleagues have demonstrated that a single stem cell derived lineage contains all of the information necessary to account for the gradual shift from neurogenesis to gliogenesis over time, it remains to be seen whether the same mechanism that operates in culture also accounts for this shift in vivo. A simple but interesting experiment would be to isolate stem cells from the postnatal subventricular zone at a time when gliogenesis predominates and assay whether they still generate neurons and then glia in culture, or whether they tend to generate only glia under conditions where stem cells from the fetal ventricular zone generate neurons first. Particularly at later times in development, when the cortex is more complicated, stem cells may rely upon environmental cues that cannot easily be recapitulated by a single clone in culture; however, even if this were the case, the paper by Qian et al. (2000) suggests that stem cells may be the architects as well as the builders of the early cortex.

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Selected Reading

Burrows, R.C., Wancio, D., Levitt, P., and Lillien, L. (1997). Neuron *19*, 251–267.

Desai, A.R., and McConnell, S.K. (2000). Development *127*, 2863–2872.

Durand, B., Gao, F.-B., and Raff, M. (1997). EMBO J. *16*, 306–317.

Furukawa, T., Mukherjee, S., Bao, Z.-Z., Morrow, E.M., and Cepko, C.L. (2000). Neuron *26*, 383–394.

Gaiano, N., Nye, J.S., and Fishell, G. (2000). Neuron *26*, 395–404.

Gao, F.B., Apperly, J., and Raff, M. (1998). Dev. Biol. *197*, 54–66.

Ikuta, K., Kina, T., Macneil, I., Uchida, N., Peault, B., Chien, Y.H., and Weissman, I.L. (1990). Cell *62*, 863–874.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D.G. (1996). Genes Dev. *10*, 3129–3140.

Kantor, A.B., Stall, A.M., Adams, S., Herzenberg, L.A., and Herzenberg, L.A. (1992). Proc. Natl. Acad. Sci. USA *89*, 3320–3324.

Morrison, S.J., Perez, S., Verdi, J.M., Hicks, C., Weinmaster, G., and Anderson, D.J. (2000). Cell *101*, 499–510.

Qian, X., Davis, A.A., Goderie, S.K., and Temple, S. (1997). Neuron *18*, 81–93.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., and Temple, S. (2000). Neuron *28*, this issue, 69–80.

Wood, W.G., Bunch, C., Kelly, S., Gunn, Y., and Breckton, G. (1985). Nature *313*, 320–322.

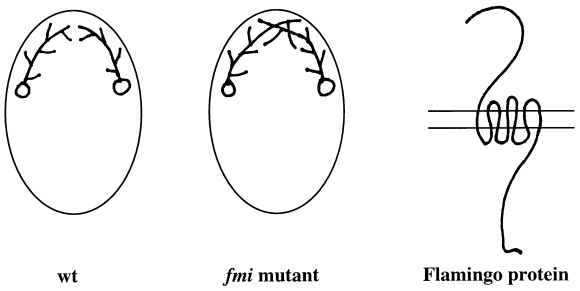
Dentritic Growth: Don't Go Says Flamingo

Neurons often extend dendritic trees that grow up to, but do not overlap with, dendritic trees of adjacent neurons. This phenomenon of dendritic tiling is most clearly apparent in the retina where each type of retinal ganglion cell covers the entire surface of the retina without any dendritic overlap. Such nonredundant coverage of the retina allows retinal ganglion cells to process visual information from nonoverlapping regions of the visual field. In a study published in this issue of *Neuron*, Gao et al. (2000) explore the basis of such dendritic filed exclusion in *Drosophila* and provide evidence that this is mediated in part by a repulsive interaction between dendrites of homologous neurons. They also show that dendritic field exclusion requires the function of Flamingo, a seven-pass transmembrane protein of the protocadherin family.

Gao et al. used the dorsal cluster neurons of the fly PNS to examine the control of dendritic development. The morphology of the six multiple dendritic (md) neurons in this cluster was revealed by selectively expressing green fluorescent protein (GFP) in these cells. The md neurons have elaborate dendritic trees that extend in different directions away from the cell body clusters. To determine if the dendritic trees of individual md neurons are influenced by signals from other cells within the same cluster, Gao et al. carried out two kinds of experiments. First, they examined the consequence of ablating a subset of neurons within a cluster on the dendritic development of the remaining neurons in the cluster and found that md neurons extend stereotyped dendritic arbors even when neighboring cells are ablated. They also found that a genetic deletion of a subset of md neurons does not affect the dendritic arborization of the remaining md neurons. Thus the dendritic pattern of md neurons is not significantly influenced by interactions with other md neurons within the same cluster.

While neurons within a cluster extend dendrites in different directions, the dorsal md neurons from either side of the embryo extend dendrites that meet at the dorsal midline. To determine if cellular interactions prevent dorsal dendrites from crossing the midline (see figure), Gao et al. ablated dorsal md neurons on one side of the embryo and examined the consequences on dendritic growth of the homologous neuron on the other side. In contrast to the effects of cell ablation on dendritic development of cells within a cluster, these ablations led to exuberant dendritic growth from homologous neurons such that they extended dendrites across the dorsal midline. These experiments suggest that a repulsive interaction at the dorsal midline between homologous dendrites is involved in restricting dendritic arbors to the appropriate regions.

The ability of dorsally extending dendrites to cross the midline was first observed by Gao et al. in the course of a mutagenesis screen to identify genes that affect dendrite development (Gao et al., 1999). One of the mutants in that screen mapped to a gene called *flamingo*



Diagrammatic Representation of Dendritic Trajectory of Dorsal md Neurons in Wild-Type and *flamingo* Mutant Flies

A *flamingo*-mediated interaction restricts dorsal dendrites from crossing the midline. Flamingo is a seven-pass transmembrane protein as depicted to the right. The resemblance between such a representation of the protein and the bird was the basis for the name "Flamingo" (Usui et al., 1999).

and was characterized by dorsal dendrites that grew toward the dorsal midline faster than wild-type dendrites and shot across the midline (see figure). The similarity between the *flamingo* mutant phenotype and the effects of md neuron ablation on homologous neurons prompted the authors to explore the possibility that the dendritic repulsion was mediated by the *flamingo* gene product. This appears to be the case since *flamingo* is expressed both in the md neurons and the adjacent epithelial cells. In addition, the *flamingo* mutant phenotype is partially rescued by expressing *flamingo* in md neurons, but not by expressing *flamingo* in epithelial cells. Thus a cell-autonomous function of *flamingo* is needed for dorsal dendrites to remain restricted to the appropriate region.

The mechanism by which Flamingo restricts dendritic growth is not known, but the presence of seven transmembrane segments in the protein is reminiscent of G protein–coupled receptors and suggests that Flamingo functions as a receptor (Usui et al., 1999). A simple model to explain the effects of *flamingo* mutations on dendritic restriction at the dorsal midline is to postulate that md neurons express both the Flamingo receptor and its ligand on dendrites, and that ligand–receptor interactions between homologous dendrites restrict dendritic growth.

So, what is a likely ligand for Flamingo? If gene family associations are an indication, Flamingo might influence dendritic development via homophilic interactions. Flamingo belongs to the protocadherin subclass of the cadherin superfamily. The protocadherins are distinct from the classical cadherins (such as N-, R-, P-, and R-cadherins) and are characterized by large ectodomains that contain multiple cadherin repreats and two laminin A globular domains, a transmembrane domain, and a cytoplasmic tail. Flamingo has nine cadherin repeats in its ectodomain, but is thus far the only member of this family that has a seven-pass transmembrane domain, suggesting that Flamingo signaling might be distinct from other protocadherins. Flamingo expressed in *Drosophila* S2 cells can mediate cell adhesion via a homophilic interaction (Usui et al., 1999), and similar interactions might contribute to dendritic exclusion. Not all Flamingo function, however, can be explained by such homophilic interactions since overexpression of *flamingo* in wild-type embryos results in *flamingo*-like overextension of dendrites, which does not fit with a simple model of Flamingo-mediated dendritic repulsion (Gao et al., 2000). Also, while the cell-autonomous rescue of the midline crossing phenotype is consistent with a Flamingo homophilic interaction model, *flamingo* expression in neurons also rescues an embryonic phenotype of rapid dendritic growth over the underlying epithelium. This suggests that Flamingo might have another ligand that is expressed in the epithelium, which normally restricts the rate of dorsally directed dendritic growth. In this regard, it is also of interest that there is evidence to suggest that Flamingo has the capacity to affect multiple, distinct signaling pathways. Flamingo has also been shown to play a role in determining planar polarity, and in this context, Flamingo appears to act downstream of Wingless/Frizzled pathway (Usui et al., 1999; Lu et al., 1999; Chae et al., 1999). In contrast, the dendritic outgrowth phenotypes appear to be independent of Frizzled.

The protocadherin gene family shot into the limelight last year with the discovery of 52 protocadherin genes in humans organized in three closely linked clusters (Wu and Maniatis, 1999, 2000). It is not known if any of the other protocadherins function in dendritic patterning, but the evidence for Flamingo raises the interesting possibility that expression of a specific protocadherin in a neuronal cell type might allow nonredundant neuropil coverage by different classes of neurons. For instance α and β retinal ganglion cells might express distinct protocadherins. A homophilic interaction might restrict overlap within one class of ganglion cells without interfering with the growth of a different class of retinal ganglion cells in the same region. In that regard, it is noteworthy that Flamingo homologs have been identified in humans (hFmi1 and hFmi2) and rodents (MEGF2, Celsr1, and mFmi1). It will be of interest to determine if these Flamingo-related genes are expressed in cell type–specific patterns in the vertebrate CNS and whether they function in dendritic patterning.

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Selected Reading

Gao, F.-B., Kohwi, M., Brenman, J.E., Jan, L.Y., and Jan, Y.N. (2000). Neuron *28*, this issue, 91–101.

Chae, J., Kim, M.J., Goo, J.H., Collier, S., Gubb, D., Charlton, J., Adler, P.N., and Park, W. (1999). Development *126*, 5421–5429.

Gao, F.B., Brenman, J., Jan, L.Y., and Jan, Y.N. (1999). Genes Dev. *13*, 2549–2561.

Lu, B., Usui, T., Uemura, T.L., Jan, L.Y., and Jan, Y.N. (1999). Curr. Biol. *9*, 1247–1250.

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R.W., Schwartz, T.L., Takeichi, M., and Uemura, T. (1999). Cell *98*, 585–595.

Wu, Q., and Maniatis, T. (1999). Cell *97*, 779–790.

Wu, Q., and Maniatis, T. (2000). Proc. Natl. Acad. Sci. USA *97*, 3124–3129.

Vesicles: Equal in Neurotransmitter Concentration but Not in Volume

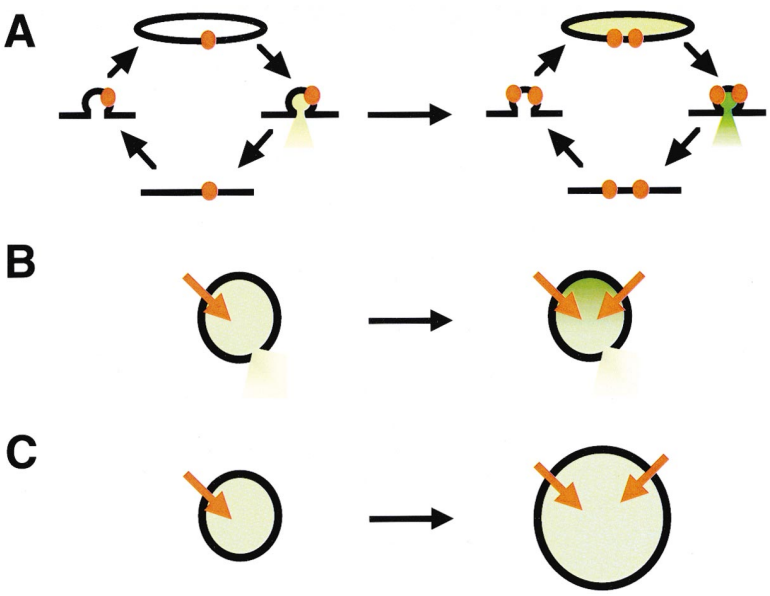
The quantal nature of neurotransmitter release is often considered to provide the elemental unit for information processing by the nervous system. Changes in synaptic strength are generally thought to involve the probability of exocytotic release and the postsynaptic response, rather than the amount of neurotransmitter per vesicle. And if postsynaptic receptors were invariably saturated by the neurotransmitter released from a single synaptic vesicle (SV), it would not matter how much each vesicle contains. However, considerable evidence indicates that postsynaptic receptors are not saturated at many synapses. Neurotransmitters also spill over to activate adjacent synapses. Thus, changes in the amount of transmitter released per vesicle (*quantal size*) clearly have the potential to alter the postsynaptic response. In addition, the amount of transmitter released per SV appears to vary considerably, even at single synapses (Liu et al., 1999; Van der Kloot, 1990). Thus, quantal release of neurotransmitter is not the invariant, elemental unit of synaptic transmission it is often considered. So what determines the amount of neurotransmitter per vesicle?

Very similar to the inwardly directed Na^+ gradient across the plasma membrane that drives neurotransmitter reuptake from the synaptic space, a H^+ electrochemical gradient drives the transport of transmitter into secretory vesicles. Transport into vesicles involves H^+ exchange, and the stoichiometry of ionic coupling influences the concentration gradients produced across the vesicle membrane. The magnitude of the outwardly directed proton electrochemical gradient and the cytoplasmic concentration of transmitter also determine the luminal concentration finally achieved. In the case of monoamines, the stoichiometry and driving force together predict concentration gradients of 10^4–10^5, and luminal concentrations in the low molar range have been demonstrated in large dense core vesicles (LDCVs). However, the dense core of LDCVs contains insoluble monoamine, which can greatly increase the amount of transmitter released without altering the concentration gradient of soluble transmitter across the vesicle membrane. Unlike LDCVs, which also contain neural peptides, SVs that release only classical small molecule transmitters do not exhibit an obvious dense core. They may contain other luminal components that reduce the solubility of the transmitter and hence increase its potential concentration, but these are certainly not as conspicuous as in LDCVs.

In this issue of *Neuron*, Bruns and colleagues use amperometry to measure the release of serotonin from the SVs and LDCVs of leech Retzius neurons (Bruns et al., 2000). Amperometry relies on the electrochemical detection of monoamines, due to their oxidation at characteristic potentials, to measure the amount of transmitter released directly and in real time, independent of the saturation or kinetics of postsynaptic receptors (Michael and Wightman, 1999). Bruns and Jahn previously used amperometry to characterize the release of serotonin from Retzius neurons and identified two modes of release that correspond to release from two distinct populations of secretory vesicles, LDCVs and SVs (Bruns and Jahn, 1995). As observed in other neurons and secretory vesicles, the two types of release differ in site and speed as well as amount (Sulzer and Pothos, 2000). In the Retzius cell, LDCVs release 10^5–10^6 molecules of monoamine over several milliseconds whereas SVs release $\sim 10^4$ molecules in less than a millisecond.

The correlation of careful morphometric analysis by electron microscopy with amperometry allows the authors of the new study to draw some very surprising conclusions (Bruns et al., 2000). First, LDCVs and SVs fill with roughly the same concentration of transmitter (270 mM). Differences in the amount of transmitter released thus primarily reflect the differences in vesicle volume, not the presence of the dense core. The correlation between monoamine release and vesicle size also holds between smaller LDCVs observed at the nerve terminal and larger LDCVs at the cell body. Second, the correlation between the size of the amperometric events and vesicle volume holds even within the population of SVs. The size distribution of small amperometric events corresponds very well to the distribution of the cubed vesicle radii, indicating that all the SVs fill to roughly the same concentration with neurotransmitter. However, the variation in large amperometric events exceeds that predicted by the variation in volume of LDCVs. In this case, fluctuation in the size of the dense core may contribute more or less insoluble monoamine that perturbs the otherwise tight correlation between soluble transmitter and vesicle size. Alternatively, vesicles may not always release their total transmitter content. Nonetheless, the results strongly suggest that all SVs and LDCVs achieve very similar luminal concentrations of releasable transmitter. These similar concentrations presumably reflect a stable equilibrium determined by the cytoplasmic concentration of transmitter, the H^+ electrochemical driving force, and the ionic coupling of the vesicular monoamine transport protein.

If vesicle filling reaches a stable equilibrium based on the transport mechanism, the number of transport proteins should make little difference to the luminal concentration of transmitter. Surprisingly, a number of observations have indicated that the level of transporter expressed even under physiological conditions limits the storage and release of both monoamines and acetylcholine (Reimer et al., 1998). How can we reconcile the apparently stable filling equilibrium achieved by secretory vesicles of leech Retzius neurons with the effect of transporter expression on quantal size? First, Retzius neurons in culture are quiescent, whereas the ongoing release of monoamines by mice in vivo may require higher rates of filling and hence higher levels of transporter expression. If the rate of SV recycling exceeds that of vesicle filling, the level of transporter expressed could dramatically affect the concentration of monoamine achieved (see figure, panel A). In this case, transporter expression affects only the rate of vesicle filling, not the equilibrium that would ultimately be achieved in quiescent neurons. The very slow turnover reported for vesicular monoamine and acetylcholine transporters (1–5/s) and the effect of activity on dopamine release by vesicular monoamine transporter 2 (VMAT2)–deficient



Mechanisms by which the Level of Transporter Expression May Influence Vesicle Filling

(A) If synaptic vesicles cycle faster than they fill with transmitter (green), increases in transporter expression (red balls), which increase the rate of filling, would result in higher luminal amounts at the time of exocytosis. If vesicles cycle more slowly than they fill, increases in transporter expression should not increase luminal amounts because filling would have already reached equilibrium before exocytosis.

(B) If synaptic vesicles exhibit a constant, nonspecific leak of neurotransmitter (the *leaky bathtub model)*, increases in the rate of filling due to increased transporter expression (red arrows) will result in higher luminal concentrations.

(C) Increases in transporter expression increase the amount but not the concentration of transmitter per vesicle by increasing vesicle volume.

mice support a role for this mechanism (Reimer et al., 1998).

Second, filling may oppose a nonspecific efflux of monoamine from the vesicle (the *leaky bathtub model*) (see figure, panel B). By increasing the amount of transmitter accumulated in the presence of a relatively fixed leak, increased transporter expression alters the equilibrium ultimately reached, not simply the rate at which it is achieved. Third, vesicles may sense filling with transmitter and shut down transport at a particular luminal concentration. Interestingly, the heterotrimeric G protein G_{o2} has been observed to associate with secretory vesicles and to inhibit VMAT2 function (Höltje et al., 2000). However, loading with L-DOPA, as well as increases in VMAT2 expression, also increases quantal monoamine release (Pothos et al., 2000), suggesting that a sensor with a specific threshold does not exist. Rather, the close correlation between variation in amperometric events and SV volume observed by Bruns and colleagues suggests a surprising alternative mechanism for the apparent uniformity of transmitter concentration.

LDCVs and SVs may fill to a limit set by osmolarity as well as to an equilibrium governed by the cytoplasmic concentration of transmitter, the driving force, and the ionic coupling of transport. Consistent with this possibility, the luminal concentration of monoamine estimated by Bruns et al. (270 mM) is very close to isotonic. But how can the amount of transmitter per vesicle be increased? If the concentration of transmitter remains constant, this predicts that the vesicles swell. Remarkably, two recent papers provide direct support for this hypothesis (see figure, panel C). Using voltammetry to measure the *concentration* of catecholamine very close to release sites on rat PC12 cells (amperometry measures total *amount* rather than concentration), Ewing and colleagues show that although L-DOPA increases the number of transmitter molecules released per quantum, it does not increase the resulting transmitter concentration (Kozminski et al., 1998). More recently, the group has provided direct evidence that LDCVs swell as the quantal size is increased by L-DOPA (Colliver et al., 2000). The halo of soluble material surrounding the dense core increases in volume. Conversely, the VMAT inhibitor reserpine reduces vesicle volume. Further, these observations suggest an explanation for the close correlation between variation in quantal size and SV volume observed by Bruns et al.—increased filling presumably distends the vesicle rather than increasing the luminal concentration of transmitter. The energy provided by the driving force that would otherwise have increased the concentration gradient across the vesicle membrane may instead distend the wall of the vesicle.

It is remarkable that secretory vesicles can increase dramatically in volume (up to 4-fold) without rupture. Changes in biogenesis have been observed to alter both synaptic vesicle volume and quantal size (Zhang et al., 1998), but the changes observed after loading monoamine cells with L-DOPA presumably involve vesicles that have already formed. Perhaps a characteristic composition of lipids helps the vesicles to accommodate such large increases in volume.

In summary, recent work indicates the potential for changes in quantal size to contribute to synaptic plasticity. However, the mechanism may involve changes in vesicle volume rather than transmitter concentration. In addition, we do not yet understand how the rate of vesicle cycling compares to filling with transmitter, and this may provide an additional constraint on quantal size. Finally, neurosecretory vesicles contain more than just neurotransmitter: what happens to other contents during the process of filling? For example, rapidly cycling synaptic vesicles contain extracellular concentrations of Na^+ and Cl^-, and we do not know the fate of these ions. These questions are particularly important because, unlike massive ionic shifts at the plasma membrane that do not perturb bulk phase concentrations in the cytoplasm or extracellular space, translocation across the vesicle membrane dramatically affects the luminal concentration of ions as well as neurotransmitter.

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Selected Reading

Bruns, D., and Jahn, R. (1995). Nature *377*, 62–65.

Bruns, D., Riedel, D., Klingauf, J., and Jahn, R. (2000). Neuron *28*, this issue, 205–220.

Colliver, T.L., Pyott, S.J., Achalabun, M., and Ewing, A.G. (2000). J. Neurosci. *20*, 5276–5282.

Höltje, M., von Jagow, B., Pahner, I., Lautenschlager, M., Hörtnagl, H., Nürnberg, B., Jahn, R., and Ahnert-Hilger, G. (2000). J. Neurosci. *20*, 2131–2141.

Kozminski, K.D., Gutman, D.A., Davila, V., Sulzer, D., and Ewing, A.G. (1998). Anal. Chem. *70*, 3123–3130.

Liu, G., Choi, S., and Tsien, R.W. (1999). Neuron *22*, 395–409.

Michael, D.J., and Wightman, R.M. (1999). J. Pharm. Biomed. Anal. *19*, 33–46.

Pothos, E.N., Larsen, K.E., Krantz, D.E., Liu, Y.-J., Haycock, J.W., Setlik, W., Gershon, M.E., Edwards, R.H., and Sulzer, D. (2000). J. Neurosci. *20*, 7297–7306.

Reimer, R.J., Fon, E.A., and Edwards, R.H. (1998). Curr. Opin. Neurobiol. *8*, 405–412.

Sulzer, D., and Pothos, E.N. (2000). Rev. Neurosci. *11*, 159–212.

Van der Kloot, W. (1990). Prog. Neurobiol. *36*, 93–130.

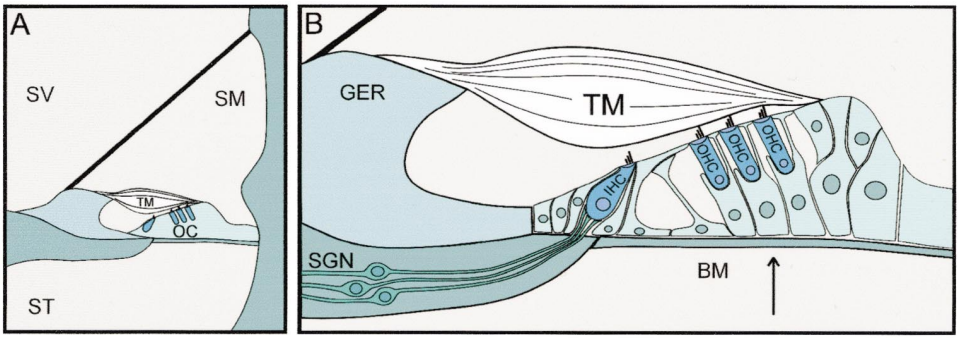
Zhang, B., Koh, Y.H., Beckstead, R.B., Budnik, V., Ganetzky, B., and Bellen, H.J. (1998). Neuron *21*, 1465–1475.

Sound Amplification in the Inner Ear: It Takes TM to Tango

Sound impinging upon a mammal's ear must travel along a wonderfully elaborate series of membranes, levers, and tubes to reach its cellular receiver within the cochlea, the mechanosensory hair cell. After coursing down the ear canal and deflecting the tympanic membrane, each compression cycle of a sound wave causes the piston-like stapes to push on a window into the cochlea, thereby increasing the pressure in a fluid-filled tube called the scala vestibuli (see figure, panel A). Increased pressure in the scala vestibuli and its neighboring tube, the scala media, pushes down on the organ of Corti, a ribbon of sensory hair cells and supporting cells that runs the length of the cochlea. The organ of Corti is suspended on the basilar membrane, a trampoline-like array of elastic fibrils that deflects under pressure, so that it bounces slightly with each cycle of sound (see figure, panel B). Adjacent to the organ of Corti, the greater epithelial ridge synthesizes and secretes a diaphanous extracellular matrix—the tectorial membrane—which is coupled at its far end to the tips of the hair cells' mechanosensory stereocilia (Kimura, 1966; Lim, 1987). Because the tectorial membrane and basilar membrane are essentially hinged at different points, there is shear between them at the level of the stereocilia. When the basilar membrane moves down, the stereocilia are pulled one way (to the left in the figure); as it moves up in the next half-cycle, the stereocilia are pushed to the right. Stereocilia deflection opens transduction channels to cause a receptor potential in all hair cells, but only the inner hair cells use their receptor potentials to modulate neurotransmitter release onto the postsynaptic spiral ganglion neurons. Although they sense the vibration, outer hair cells do not seem to make functional synapses on postsynaptic neurons.

But then something magic happens. Somehow the outer hair cells respond to the vibration of the basilar membrane by pushing back on it, exerting force with just the right amplitude and phase to amplify the vibration, especially for faint sounds, by 100-fold or more. The movement of the basilar membrane is amplified from hundredths of nanometers to around a nanometer for the quietest perceptible sounds, from a nanometer to several nanometers at conversational level, but not much at all for loud sounds. In a healthy ear, movement is therefore not linearly proportional to sound level, but shows a compressive nonlinearity. Immediately postmortem, or even with acute occlusion of cochlear blood flow, the active amplification disappears and the basilar membrane movement is proportional to sound level. Moreover, outer hair cells in each region of the long organ of Corti only amplify sound of a particular frequency, so that each region is exquisitely tuned to a characteristic frequency (CF) and not to other frequencies. Inner hair cells then sense the amplified vibration, and send a frequency-specific signal to spiral ganglion neurons of the eighth nerve (Hudspeth, 1997).

A new study from Guy Richardson, Ian Russell, and their colleagues (Legan et al., 2000 [this issue of *Neuron*]) explores the role of the tectorial membrane in active tuning of the basilar membrane. Over the last decade, Richardson's group has made the major contribution to our understanding of the structure of the tectorial membrane (TM). The TM is composed of collagens—types II, V, and IX—and of collagenase-insensitive glycoproteins. The collagen fibrils run radially across the TM and are embedded in a striated-sheet matrix composed of two types of 7–9 nm filaments. Two of the major glycoproteins—α- and β-tectorin—were purified from chick TM and cloned from chick and mouse by Richardson's group (Legan et al., 1997). α-tectorin is a large protein with an N-terminal entactin G1 domain, a central region with five full or partial von Willibrand factor type D repeats, and a C-terminal zona pellucida domain (Legan et al., 1997). Mutations in human α-tectorin underlie two dominantly inherited human nonsyndromic deafnesses, DFNA8 and DFNA12 (Verhoeven et al., 1998). Richardson's group has now generated a targeted deletion in the mouse α-tectorin gene (*Tecta*), and finds, remarkably, that the mouse has no functional TM. This genetic decoupling of basilar membrane vibration and outer hair cell feedback provides a physiological system in which to study the amplification.

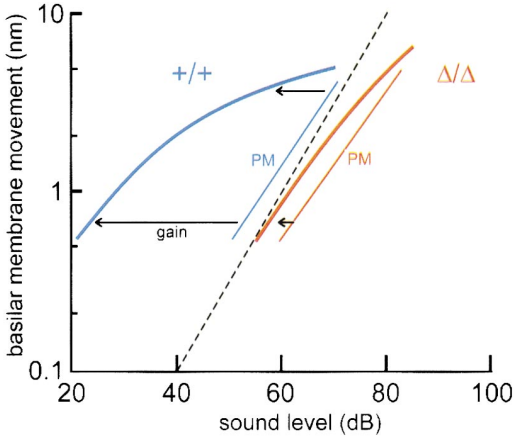


Structure of the Mammalian Cochlea

(A) The organ of Corti (OC) separates the scala vestibuli (SV) and scala media (SM) from the scala tympani (ST), and moves in response to pressure differences between them.

(B) The tectorial membrane (TM) is synthesized by cells of the greater epithelial ridge (GER) and contacts stereocilia of outer hair cells (OHC). Inner hair cells (IHC) respond to sound and make synapses with spiral ganglion neurons (SGN). Hair cells and supporting cells ride on the basilar membrane (BM), whose motion is measured by a laser interferometer (arrow).

Mice homozygous for deletion of 96 amino acids in the entactin G1-like domain (*Tecta*$^{\Delta/\Delta}$) have no α-tectorin in their cochleas detectable by immunoblot, suggesting the mutant protein is rapidly degraded. While the TM is made in *Tecta*$^{\Delta/\Delta}$ mice, within a few weeks after birth it is no longer attached to the region of greater epithelial ridge, nor to the hair cells, but instead floats away to the far reaches of the scala media. The otolithic membrane, a related structure in vestibular organs that contains α-tectorin, is similarly absent. Collagens remain in the TM of *Tecta*$^{\Delta/\Delta}$ mice, but additional glycoproteins—β-tectorin and otogelin—are missing as well, suggesting that their stability within the TM depends on α-tectorin. Finally, the striated-sheet matrix of the TM is missing and the collagen fibrils are disorganized; thus, α-tectorin is required for, and probably forms a major part of, the striated-sheet matrix.



Relation between Sound Pressure Level and Basilar Membrane Motion

In wild-type mice (+/+; blue lines), cochlear amplification boosts the vibration caused by quiet sounds, but amplification disappears postmortem (thin line; PM), leaving a response close to the passive, linear prediction (dashed line). In *Tecta*$^{\Delta/\Delta}$ mice (Δ/Δ; red lines), the response is nearly linear at all sound levels; both before and after death the relation is close to passive.

What happens to cochlear tuning and amplification in the absence of a functional TM? In *Tecta*$^{\Delta/\Delta}$ mice, amplification is basically gone. Basilar membrane motion still shows some tuning, presumably reflecting the passive mechanical resonance of the basilar membrane. The CF is the same as expected, so that the passive tuning seems not to depend on the mass and stiffness of the TM. However, the 100-fold amplification and the very sharp tuning at low sound levels is gone. The movement with increasing sound level is nearly linear, and it changes little after death of the animal. These are all to be expected if deflection of outer hair cell stereocilia by the TM is a critical step in the amplification.

On the other hand, outer hair cells still respond to sound in *Tecta*$^{\Delta/\Delta}$ mice, as assessed by recording the extracellular cochlear microphonic potential, so the mechanical coupling to stereocilia must be different. With no TM, the stereocilia are apparently moved by fluid drag: even for nonamplified frequencies, the cochlear microphonic is about 10-fold smaller, indicating inefficient coupling, and the phase in the *Tecta*$^{\Delta/\Delta}$ mice leads that of wild-type by 90°, as expected for a velocity-dependent drag.

Yet even adjusting for inefficient coupling, the amplification is absent in *Tecta*$^{\Delta/\Delta}$ mice. Legan et al. (2000) suggest two possibilities why. One is that the phase lead associated with fluid coupling upsets the critical timing needed for cycle-by-cycle feedback—that the outer hair cell is pushing too early in each cycle to boost the vibration. The other is that the TM, in addition to bringing the vibration of sound to the stereocilia, is a structural part of the feedback—that the outer hair cells push back *against* the TM to move the basilar membrane. Probably both are true in degree.

For all its elegance, the *Tecta*$^{\Delta/\Delta}$ mouse does not really address one of the most interesting questions in cochlear amplification: what is the motor? Outer hair cells display an extraordinary type of electromotility, expanding or contracting along their length in microseconds when their membrane potential is changed (Dallos, 1992). Their membranes are packed with a newly identified protein, prestin, that appears to be the motor protein (Zheng et al., 2000). This motility, apparently unique to

outer hair cells, must be doing something important. On the other hand, there are concerns that the outer hair cell membrane potential cannot change quickly enough to drive the electromotility cycle-by-cycle. Independently, the stereocilia themselves can produce a small but fast movement when deflected, which is thought to result from calcium binding to the transduction channels after they open and which does not rely on a receptor potential. It has been proposed that this movement instead powers the cochlear amplifier, by pushing directly back on the TM (Howard and Hudspeth, 1988). In support of this idea, rapid stereocilia movement is observed in lower vertebrates that can display sharp frequency tuning but lack outer hair cell motility (Ricci et al., 2000). We await the next generation of transgenic mice, with putative motors deleted, to resolve the issue.

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Selected Reading

Dallos, P. (1992). J. Neurosci. *12*, 4575–4585.

Hudspeth, A. (1997). Curr. Opin. Neurobiol. *7*, 480–486.

Kimura, R.S. (1966). Acta oto-Laryng. *61*, 55–72.

Howard, J., and Hudspeth, A.J. (1988). Neuron *1*, 189–199.

Legan, P.K., Rau, A., Keen, J.N., and Richardson, G.P. (1997). J. Biol. Chem. *272*, 8791–8801.

Legan, P.K., Lukashkina, V.A., Goodyear, R.J., Kössl, M., Russell, I.J., and Richardson, G.P. (2000). Neuron *28*, this issue, 273–285.

Lim, D.J. (1987). Hear. Res. *28*, 9–21.

Ricci, A.J., Crawford, A.C., and Fettiplace, R. (2000). J. Neurosci. *20*, 7131–7142.

Verhoeven, K., Van Laer, L., Kirschhofer, K., Legan, P.K., Hughes, D.C., Schatteman, I., Verstreken, M., Van Hauwe, P., Coucke, P., Chen, A., et al. (1998). Nat. Genet. *19*, 60–62.

Zheng, J., Shen, W., He, D.Z., Long, K.B., Madison, L.D., and Dallos, P. (2000). Nature *405*, 149–155.